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I HEREBY CERTIFY that annexed hereto is a true copy of documents filed in connection with the following patent application:

Application No. 2003/0773

Date of Filing 17 October 2003

Applicant TEAGASC, THE AGRICULTURE AND FOOD DEVELOPMENT AUTHORITY AND UNIVERSITY COLLEGE CORK, a Statutory Body Corporate under the Agriculture (Research, Training and Advice) Act, 1988, a body Corporate, incorporated by Royal Charter respectively, of 19 Sandymount Avenue, Dublin 4, Ireland, and College Road, Cork, Ireland.

Dated this 20 day of October 2004.

An officer authorised by the
Controller of Patents, Designs and Trademarks.

030773

Form No.1

REQUEST FOR THE GRANT OF A PATENT

Patents Act, 1992

The Applicant(s) named herein hereby request(s)

☒ the grant of a patent under Part II of the Act

☐ the grant of a short term patent under Part III of the Act on the basis of the information furnished hereunder

1. Applicant(s)

Name: **TEAGASC, The Agriculture and Food Development Authority and UNIVERSITY COLLEGE CORK**

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Description/Nationality : **a Statutory Body Corporate under the Agriculture (Research, Training and Advice) Act, 1988**
a body Corporate, incorporated by Royal Charter respectively.

2. Title of Invention:

USE OF PROBIOTIC BACTERIA IN THE TREATMENT OF INFECTION

3. Declaration of Priority on basis of previously filed application(s) for same invention (Sections 25 & 26)

Previous Filing Date	Country in or for which filed	Filing No.

4. Identification of Inventor(s):

Name(s) of person(s) believed by applicants to be the inventor(s) address:

5. Statement of right to be granted a patent (Section 17(2) (b))

Date of assignment from inventors:

6. Items accompanying this Request - tick as appropriate

(i) ☒ prescribed filing fee

(ii) ☒ specification containing a description and claims
☐ specification containing a description only
☐ Drawings to be referred to in description or claims

(iii) ☐ An abstract

(iv) ☐ Copy of previous application(s) whose priority is claimed

(v) ☐ Translation of previous application whose priority is claimed

(vi) ☒ Authorisation of Agent (this may be given at 8 below if this request is signed by the applicant(s))

7. Divisional Application(s)

The following is applicable to the present application which is made under Section 24 -

Earlier Application No:
Filing Date:

8. Agent

The following is authorised to act as agent in all proceedings connected with the obtaining of a patent to which this request relates and in relation to any patent granted -

Name	Address
TOMKINS & CO.	**5 Dartmouth Road, Dublin 6.**

9. Address for Service (if different from that at 8)

TOMKINS & CO., at their address as recorded for the time being in the Register of Patent Agents.

Signed Name(s): M. Collins
by:
Capacity (if the applicant is a body corporate):

Date: **17 October 2003**

KPM4307

Title

Use of Probiotic Bacteria in the Treatment of Infection

Field of the Invention

The present invention relates to the use of live cultures of non-pathogenic, probiotic bacteria or supernatants of such cultures in the treatment of bacterial infections, particularly localised infections. In particular the invention relates to the use of live cultures of *Lactococcus lactis* or the supernatant from such cultures in the treatment of mastitis. This invention concerns the exploitation of food-grade "harmless" probiotic bacteria for the treatment of infectious diseases (or localised infections) in humans and animals. In particular the treatment involves the application of a non-pathogenic lactic acid bacterium to the infected animal/human which results in relief in clinical symptoms of the infection/disease.

Background to the Invention

The notion of "friendly" bacteria contributing to good health and well-being was first proposed almost a century ago by Prof. E. Metchnikoff, but it is only in the last two decades that the potential health promoting role of some bacteria has been fully appreciated. Probiotic therapy uses bacterial interference and immunomodulation in the control of several infectious, inflammatory, and immunologic conditions. For instance, there is growing evidence to suggest that while an impoverished or absent gastrointestinal (GI) tract microflora can lead to digestive problems like hypoallergenic intolerance; recolonisation by "friendly" bacteria has the capacity to restore oral tolerance and regain the development of a balanced immune system (Alvarez-Olmos and Oberhelman, 2001; Cross, 2002). While the intricacies of signalling between the *de novo* colonisers and the immune system are not fully elucidated, it is believed that modulation of the immune response probably occurs through one or a combination of the following mechanisms (Cross, 2002):

1. Localised lactic acid production by probiotics, which may limit the growth of pathogens.
2. Production of anti-pathogenic substances by the probiotic strain e.g. bacteriocins, which are potent bactericidal compounds.

3. Limitation of colonisation by competing for colonisation sites- "competitive exclusion".
4. Production of immunomodulatory signals by the probiotic strain that stimulate the host immunity sufficiently to afford a degree of enhanced protection.

Lactic acid bacteria (LAB), including members of the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pedicococcus* and *Streptococcus* have been used for millennia in the production of fermented foods. As a result of their history as harmless bacteria, these microorganisms are considered as GRAS (Generally Regarded As Safe) for many applications, including human and animal consumption. In recent years, there has been extensive research into the use of LAB in the control of pathogenic microorganisms, and as health-promoting agents or "probiotics".

To date, probiotic therapy has mainly been exploited in the treatment of gastrointestinal problems. While initially based on hearsay and tradition, the peer-approved scientific evidence now supporting the protective role of probiotics and in particular the LAB Lactobacilli, in the GI tract is immense. Multiple antimicrobial properties of probiotics have been suggested as potential protective factors in the human digestive system against microorganisms such as *Escherichia coli*, *Helicobacter pylori*, *Salmonella* and *Listeria* species (Alvarez-Olmos and Oberhelman, 2001). For instance, mice which were fed *Lactobacillus acidophilus*, *Lb. casei* or a combination of both, prior to oral challenge with *Salmonella typhimurium*, had reduced pathogen translocation to the spleen and liver, compared with control mice. This resulted in increased survival of mice in the probiotic-fed groups, particularly in the group fed both strains. This study also demonstrated that in the probiotic-fed groups, macrophages had increased phagocytic activity (Perdigon *et al.*, 1990a).

The protective effect of *Lb. casei* against *S. typhimurium*, *E. coli* and *Shigella sonnei* has also been investigated in mice. Increased protection from oral challenge with the aforementioned pathogens was observed when mice were pre-fed *Lb. casei*. Additionally, increased IgA levels were observed, and probiotic-fed mice challenged with *Shigella* had increased anti-*Shigella* antibody titres in the serum and GI tract compared to the control group (Perdigon *et al.*, 1990b; 1991).

A growing body of evidence, therefore, links increased anti-microbial protection with the enhancement of appropriate immune responses by probiotics. Recently, research has investigated the use of immunomodulatory probiotics as

protective agents in the GI tract, and also at other mucosal surfaces. In one such study, mice pre-fed *Lb. casei* were subjected to an aerosolised challenge of *Pseudomonas aeruginosa* (Alvarez *et al.*, 2001). The results demonstrated that probiotic feeding increased the clearance rate of *P. aeruginosa* from the lungs, up-regulated the phagocytic capacity of the alveolar macrophages and increased the levels of IgA in the serum and broncho-alveolar lavage fluid. It is apparent from these results that probiotic feeding can influence immuno-responses in the respiratory tract tissues and that this effect is sufficient to afford protection against bacterial respiratory tract pathogens. Furthermore, *Lb. rhamnosus* GR-1 and *Lb. fermentum* RC-14 are well recognised as therapeutic agents in the prevention and treatment of urogenital infections in women. Restoration of a healthy and normal vaginal flora occurs following local application of Lactobacilli, demonstrating that probiotics delivered locally, as well as those delivered by the oral route, can provide enhanced protection against pathogens (Reid *et al.*, 2001; Gardiner *et al.*, 2002). Thus, the areas of potential use of probiotics has expanded rapidly in recent decades, and now includes prevention and treatment of diarrhoeal diseases in adults and children, prevention of vaginitis and urinary tract infection in adults, food allergy prevention, and antitumor action in the gut, bladder and cervix (Cross, 2002).

Apart from the obvious benefits of using GRAS organisms for the latter purposes, using Gram positive bacteria like lactococci, lactobacilli and streptococci has the added advantage that the cell wall of Gram positive bacteria has been shown to act as an immune-response activator. Another major attraction of using lactic acid bacteria as therapeutic agents stems from their ability to produce bacteriocins, potent anti-microbial peptides (Ross *et al.*, 1999). These peptides kill other microorganisms rapidly by destroying or permeating the microbial membrane and impairing the ability to carry out anaerobic processes. Because of their mode of action, these peptides are unlikely to face the same antimicrobial resistance mechanisms that limit current antibiotic use.

Nisin was the first identified bacteriocin derived from fermentation of a lactic acid bacterium, *Lactococcus lactis*. It is approved for use as a food preservative in the United States, and was awarded GRAS status in the U.S. Federal Register in 1988. It is also approved as a natural food preservative by more than 40 other countries as well as the World Health Organisation and the European Union. In addition to its use as a food additive to inhibit spoilage organisms and pathogens, several studies have

investigated its use as a therapeutic agent, in the treatment of such diverse diseases as acne, human gastrointestinal infections and bovine mastitis (Blackburn *et al.*, 1994; Sears *et al.*, 1995). It is currently used as a component of a commercial teat-dip product (CONSEPT ®, Babson Bros.).

Lacticin 3147 is a broad-host range bacteriocin also produced from a lactococcal strain, *L. lactis* DPC3147. It was first identified in an isolate obtained from an Irish kefir-like grain that had been used domestically for the production of buttermilk. It kills all Gram-positive bacteria tested to date, including high profile antibiotic resistant pathogens such as methicillin resistant staphylococci, vancomycin resistant enterococci, and penicillin resistant pneumococci (Galvin *et al.*, 1999) in addition to food poisoning organisms such as *Listeria monocytogenes* and *Clostridium botulinum* (Ross *et al.*, 1999). Similar to nisin, it is a member of the family of bacteriocins termed lantibiotics. It is a two-component bacteriocin, with both components required for full activity. Its mode of action involves the formation of pores which, by damaging the membrane of sensitive cells, leak potassium and phosphate ions. Importantly, lacticin 3147 has advantages over nisin as a choice of therapeutic agent, including its effectiveness over a broad pH range (nisin is most effective at acid pH), which suggests additional possibilities in non-acid foods and in biomedical applications (Ross *et al.*, 1999). Lacticin 3147 has already been exploited for a wide range of applications, including use as a powdered biopreservative (Morgan *et al.*, 1999) and in the treatment of bovine mastitis (Ryan *et al.*, 1999; Twomey *et al.*, 2000).

Nutritional competition is established as an important mechanism by which probiotics exert their effect. Suppressive factors such as bacteriocins and toxicity of end metabolic products have also been implicated (Alvarez-Olmos and Oberhelman, 2001; Cross, 2002).

Mastitis is defined as inflammation of the udder and is indicated by increases in Somatic Cell Count (SCC). The SCC is an indication of the levels of neutrophils in the milk, which in turn is an indication of the presence of infection. A normal udder quarter is free from pathogenic bacteria, has very few neutrophils in the milk, and thus, a low SCC (<0.2 x 10^6/ml SCC). A rise in SCC usually indicates the presence of an infection.

When a cow has clinical mastitis, the affected quarter may have obvious signs of inflammation- heat, pain and swelling, and the cow may have an elevated body

temperature. The SCC is raised above 0.2 x 10^6/ml and pathogens may (specific clinical) or may not (non-specific clinical) be detectable. Quarters are also considered clinical, if the milk is visibly abnormal- e.g. clots present, even though there may be no clinical signs on palpation. Clinical mastitis can be classified on the basis of the appearance of the milk from affected quarters. A clinical or subclinical infection is referred to as "Chronic" if it has persisted over a long period and does not respond to antibiotic treatment. Clinical chronic cases are easily identified by the milker. In subclinical cases, the affected udder appears normal but the milk has an elevated SCC (>0.2 x 10^6/ml) and pathogens are usually present in the milk. Subclinical chronics are only identified by repeated sampling and laboratory analysis. An EC Council Directive sets out regulations for the hygienic production of milk and dairy products.

Chronic cases are treated routinely with antibiotic treatment. There are cases, however, that do not respond to antibiotic treatment, or cases which respond briefly, and then reoccur, even following repeat administration of antibiotic. Repeated antibiotic administration results in milk loss, as milk must be withheld from the creamery until the milk is free of antibiotic residues.

We have investigated the use of a live culture of *L. lactis* 3147 in the treatment of bovine mastitis. Use of the bacteriocin-producing culture in place of a concentrated lacticin preparation has certain advantages. Firstly, the producing organism, *L. lactis* is GRAS, and was isolated from a food source. The use of the live culture for the treatment of mastitis, can be viewed as a double-edged sword – not only is bacteriocin produced in a natural and stable manner, but the culture should also compete with pathogens for colonisation of the teat. Additionally, other antimicrobial substances, such as organic acids, free fatty acids, ammonia, and hydrogen peroxide may also be produced as end products of metabolism. It was found that infusion of *L. lactis* 3147 into the teat duct resulted in a short-lived rise in SCC, with a concomitant reduction or elimination of pathogens, and followed by a dramatic improvement in both the clinical appearance of milk and in the quality of the milk.

Object of the Invention

It is an object of the invention to provide an improved method of combating infectious diseases, particularly localised infections such as mastitis. It is a further object to provide a pharmaceutical composition or a method of treatment for such

infection which does not involve the use of antibiotics and which utilises the properties of non-pathogenic and food grade bacteria.

Summary of the Invention

According to the present invention there is provided use of a live culture of a non-pathogenic food-grade probiotic bacterium in the treatment of infectious diseases. The infectious disease may be a localised infection of the skin, including an infected wound, a urinary tract infection or mastitis.

The probiotic bacterium may be a non-pathogenic lactic acid bacterium. The lactic acid bacterium may be a Lactococcus strain. One suitable Lactococcus strain is *Lactococcus lactis* DPC3147.

In an alternative embodiment the invention also provides the use of the supernatant of a live culture of a non-pathogenic food-grade probiotic bacterium in the treatment of infectious disease. The disease and the bacterium may be as described above.

The invention also provides the use of a live culture of a non-pathogenic food grade probiotic bacterium or the supernatant of a live culture of a non-pathogenic food-grade probiotic bacterium in the preparation of a medicament for the treatment of infectious diseases of humans and animals.

In a still further embodiment the invention provides a pharmaceutical composition comprising a pharmaceutically effective amount of a non-pathogenic live culture of a food-grade probiotic bacterium or a pharmaceutically effective amount of the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium together with a pharmaceutically acceptable carrier or diluent.

Also provided is a method of treatment of infectious diseases comprising administering to a subject a pharmaceutically effective amount of a non-pathogenic live culture of a food grade probiotic bacterium or the supernatant of a non-pathogenic live culture of a food grade probiotic bacterium. The method is suitable for the treatment of mastitis.

It could not be predicted that these live cultures would be effective in disease treatment for many reasons. Firstly, in most probiotic applications the organism used is one which is normally found at the site of treatment, and most such applications are for the treatment of gastrointestinal tract problems. The effectiveness of *L. lactis* is particularly surprising in that it is not an enteric organism and moreover it is not found

normally in the udder. Furthermore there has never been a suggestion in the prior art that non-pathogens could be successfully used to stimulate the immune system for these applications.

In another embodiment the invention provides use of a live culture of a non-pathogenic food grade probiotic bacterium or the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium in a method of stimulation of the immune system for these applications.

In a further embodiment the invention provides a method of accelerating improvement in the quality of milk from cows with mastitis whereby the status of Somatic Cell Count and Total Bacteria Count is rapidly brought within the range set by the EC Council Directive Regulations.

Brief Description of the Drawings

Figure 1A and B: Somatic Cell Count values and bacterial counts in Quarters of treated animals.

Figure 2A. Appearance of milk from Cow 1154LF sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 2B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 1154LF sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 3A. Appearance of milk from Cow 1178LH sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 3B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 1178LH sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 4A. Appearance of milk from Cow 1850RF sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147. Samples shown on Day 0 and 9 days post-infusion.

Figure 4B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 1850RF sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 5A. Appearance of milk from Cow 1163RH sampled pre- and post-infusion of *Lactococcus lactis* DPC 31.

Figure 5B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 1163RH sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 6A. Appearance of milk from Cow 1184RF sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 6B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 1184RF sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 7A. Appearance of milk from Cow 14LH sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 7B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 14LH sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 8A. Appearance of milk from Cow 717RF sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 8B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 717RF sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 9A. Appearance of milk from Cow 264LF sampled pre- and post-infusion of *Lactococcus lactis* DPC 3147.

Figure 9B. Graphs of Somatic Cell Count and Clinical Score in milk from Cow 264LF sampled pre- and post-infusion of *L. lactis* DPC 3147.

Figure 10. Leukocyte numbers in individual quarters before and after treatment with either *L. lactis* DPC3147 (Cow 273RH and Cow 1850RF); or sterile broth (Cow 273 LH).

Figure 11. Leukocyte numbers in individual quarters in Cow 1803 before and after treatment with either *L. lactis* DPC3147 (RH); intr-mammary antibiotic (LF); cell-free supernatant (LH) or untreated control (RF).

Figure 12A. Superoxide Anion Production by PMN in each of four quarters in one cow (Cow 1803) before and after treatment with either *Lactococcus lactis* DPC3147 (RH); intra-mammary antibiotic (LF); cell-free supernatant (LH) or untreated control (RF).

Figure 12B. Levels of superoxide anion fluorescence intensity in each of the four quarters of Cow 1803 before and after treatment with either *L. lactis* DPC3147 (RH); intra-mammary antibiotic (LF); cell-free supernatant (LH) or untreated control (RF).

Figure 13. Somatic cell counts in the four quarters of Cow 1803 after infusion with either *L. lactis* DPC3147 (RH); intra-mammary antibiotic (LF); cell-free supernatant (LH) or untreated control (RF). Day 0 = pre-infusion.

Figure 14. Leukocyte numbers in individual quarters in Cow 1137, Cow 1852 and Cow 1570 before (Day 0) and after treatment with either live *L. lactis* DPC3147, dead *L. lactis* DPC3147, sterile saline or untreated control.

Figure 15. PMN numbers in individual quarters before and after the various treatment with either *L. lactis* DPC3147 (*L. lactis*); *L. lactis* DPC5399 (Bact neg); *Lb. plantarum* DPC4922 (plantarum) or untreated controls.

Figure 16. Relative proportional post-treatment increase in milk-derived PMN relative to pre-treatment in Cow 1163, Cow 1171 and Cow 1181 respectively.

Figure 17. The effect of infusing different preparations of *L. lactis* DPC3147 on PMN numbers from Cow 275, Cow 1134 and Cow 2810. Quarters of each cow were treated with an overnight culture of *L. lactis* DPC3147 (O/N); a freeze-dried culture of *L. lactis* DPC3147 (FD) or left untreated. PMN numbers were then analysed from each quarter over two days.

Detailed Description of the Invention

Materials and Methods

Preparation of infusion mixture

Lactococcus lactis DPC3147 was isolated previously from a kefir grain. It was routinely propagated at 30°C in M17 broth (Difco Laboratories, Detroit, USA) supplemented with 0.5% glucose or lactose. This culture (3ml, ~10^9 cfu ml^{-1}) was either used directly, or an infusion mixture was prepared in the following way: 2ml of an overnight culture of *L. lactis* DPC3147 were mixed with 3ml sterile water for injection (Antigen Pharmaceuticals) to produce a working culture concentration of approximately 10^9 cfu ml^{-1}. Control infusion mixtures included uninoculated broth, incubated overnight at 30°C and then diluted in a similar fashion to the culture.

Infusion techniques

The diluted *L. lactis* DPC3147 cultures were infused directly into the teat sinus via the streak canal. The culture was inoculated to a depth of 17mm using a syringe with a blunted smoothed tip to prevent injury to the teat. Infusion of the mixture was usually performed after the evening milking. For the six chronic disease cases, a single application of 3 mls of undiluted culture was performed. For the nine clinical mastitis cases, infusion was performed twice at a 24h interval for seven quarters (Cow 1163RH, 14LH, 717RF, 1154LF, 1850RF, 1184RF, 1176LH), twice with a 72h interval for one quarter (1178LH) and four times for Cow 264LF, with 24h between and first and second infusions, 84h between the second and third infusion and 48h between the third and fourth infusions (Appendix 1-9). 5ml of the infusion mixture

prepared as described above were used when infusing the quarters with clinical mastitis.

Selection of cows for treatment

Before *in vivo* experiments commenced, foremilk quarter samples were collected in an aseptic manner from all prospective cows and these were screened for mastitis-causing pathogens by streaking 10 µl on separate quadrants on the surface of ABA plates and incubating for 16h at 37°C. Somatic cell counts (SCC) or CMT results were also determined for each quarter before treatment. Previous history of infection was also considered during selection. Six udder quarters from 4 cows with a history of chronic infection were initially selected for treatment. Nine quarters from 9 cows with newly acquired clinical infections were also treated subsequently.

Bacterial enumeration

Eighteen hours post-infusion, foremilk samples were taken in an aseptic manner for microbiological analysis from all of the treated quarters. One hundred microlitres of each milk sample was streaked on the surface of an Aesculin blood agar plate (ABA) containing blood agar base No. 2 (Oxoid), supplemented with 7% citrated whole calf blood and 0.1% aesculin (Sigma, St. Louis, MO, USA) and incubated for 24h at 37°C. Following incubation, colonies were enumerated and identified on the basis of haemolytic activity and colony appearance on ABA. One hundred microlitres of each milk sample was also streaked on the surface of a M17 agar plate supplemented with 0.5% glucose or lactose and incubated overnight at 30°C.

Assay for Bacteriocin Activity

The antimicrobial activity of *L. lactis* colonies was assessed against *L. lactis* HP, using the agar well diffusion assay described previously (Parente and Hill, 1992). Additionally, colonies isolated on GM17 or LM17 from milk were selected randomly, purified and assayed to confirm isolation of *L. lactis* DPC3147 from udder quarters.

Immunological studies

Two independent studies were performed to investigate the effect of *L. lactis* DPC 3147 on the immunity of the cow. In the first study, two cows were used, an uninfected animal (Cow 273) and an infected animal with high SCC (Cow 1850).

One quarter of Cow 273 was infused with sterile broth as a control (Left Hind, LH) and one quarter was infused with the *L. lactis* culture (Right Hind, RH). One quarter of Cow 1850 was also infused with the *L. lactis* culture (RF). Milk samples were taken immediately prior to intramammary infusion (Day 0) to determine baseline levels of leukocyte subpopulations. Milk samples were also collected 16 hours following treatment (Day 1). In a second study, an infection-free cow with SCC counts in all udder quarters of < 100, 000/ml was chosen for immunological studies. One quarter (RH) was inoculated with the *L. lactis* DPC3147 mixture, a second quarter (LH) was infused with cell-free supernatant from an overnight culture of *L. lactis* DPC3147, a third quarter (LF) was infused with the contents of one intra-mammary antibiotic syringe containing 250mg of Neomycin Sulphate, 100mg of Procaine Penicillin, 10mg of Oxytetracycline Hydrochloride and 10mg of Prednisolone (Multimast L.C., Bimeda Ltd., Dublin) and one quarter (RF) was left untreated. Milk samples were taken just prior to intra-mammary injections (Day 0) and also 24 and 48 hours following treatment. All samples from both trials were stored at room temperature following milking and were analysed within three hours of collection. Neutrophils and lymphocytes were identified using a combination of bovine-specific antibodies (BN15.6 and CD3 respectively) and precise gating techniques in flow cytometry. Superoxide anion production assays were performed to assess the functional activity of neutrophils. In the second trial, milk samples were also taken every 24h for up to 7 days to monitor the Somatic Cell Count (SCC).

Effect of using dead cells

An experiment was performed to investigate the effect of infusing dead *L. lactis* DPC3147 cells on the immune response of cows. Three low SCC cows were selected (Cow 1852, 1135, and 1570) and the teats were randomised and infused with either live lactococci, dead lactococci or sterile saline. The live lactococci infusion mixtures were prepared as described above. The preparation of dead cells was prepared by growing *L. lactis* DPC3147 in LM17 overnight as described above, and then treating by boiling at 100°C for 10 min. Following boiling, the bacteria were plated on LM17 agar and incubated overnight to confirm lack of viability. The dead culture was mixed with sterile water for injection in a ratio of 2:3, and this mixture was used for infusion as described above. Similarly, sterile saline was also mixed with sterile water for injection and used as a control infusion mixture. A fourth quarter was left

untreated in each cow as a negative control. Milk samples were taken just prior to intra-mammary injections (Day 0) and on Days 1, 3, 6, 7 and 10 following treatment. All samples were then analysed for immunological activity as described above.

Effect of using other Lactic Acid Bacteria

Lactobacillus plantarum DPC4922 was grown anaerobically at 37°C in MRS medium. *L. lactis* DPC5399, a derivative of *L. lactis* DPC3147, which is incapable of producing bacteriocin (Bac-), was grown in an identical manner to *L. lactis* DPC3147 (as described above). Three cows (Cow 1163, Cow 1171 and Cow 1181) were selected to investigate the effects of infusing potential probiotic bacterial strains on the immune response of the mammary gland of cows with low somatic cell counts. Three cows were used to monitor the immune responses amongst different animals. Each quarter received a different treatment, with the treatments randomised amongst teats.

Milk samples were taken just prior to intramammary injections (Day 0), to determine baseline levels of leukocyte subpopulations. Milk samples were also collected 1, 2, 3, 7 and 10 days following treatment. All samples were stored at room temperature following milking and were analysed within three hours of collection. Neutrophils and lymphocytes were identified using a combination of bovine-specific antibodies and flow cytometry.

Preparation of freeze dried L. lactis DPC 3147.

LM17 (200ml) was inoculated with a 1% inoculum of *L. lactis* DPC3147 and incubated overnight at 30°C. The cells were then harvested by centrifugation at 4°C and 4500rpm in a Sorvall RC *3Cplus* centrifuge. The supernatant was removed and the cells were resuspended in ~150ml sterile distilled water. The cells were then harvested again by centrifugation and the pellet was resuspended in ~100ml sterile distilled water. The resuspended cells were then freeze-dried to a powder preparation overnight using a Modulyo Freeze Dryer (Edwards). A sample of the resulting powder was then resuspended in sterile distilled water as a 10% solution and bacteria were enumerated by plating dilutions on LM17 agar and incubating overnight at 30°C. Appropriate amounts of powder were then added to 5ml of sterile water for injection such that the final concentration was equivalent to 10^9 cfu ml^{-1} approximately. Powder resuspended in this way was then used as an infusion mixture.

Comparison of different preparations of L. lactis DPC3147

An infusion mixture containing resuspended freeze-dried *L. lactis* DPC3147 cells was prepared as described above. This mixture, and a standard infusion mixture (diluted broth culture) were then infused randomly into teats of three different cows. Three different cows were used to allow for variation between different cows in immunological response. One quarter in each cow was left untreated as a negative control. Milk samples were taken just prior to intramammary injections (Day 0), to determine baseline levels of leukocyte subpopulations. Milk samples were also collected at 24h and 48h following treatment. All samples were stored at room temperature following milking and were analysed within three hours of collection. Neutrophils and lymphocytes were identified as outlined above.

Comparison of L. lactis treatment with antibiotic therapy

A trial was performed to assess the efficacy of treatment with *L. lactis* DPC3147 in comparison to a commonly used intra-mammary antibiotic containing amoxycillin (200mg) and clavulanic acid (50mg), (Synulox, Pfizer animal Health). 24 infected quarters in 12 cows were used, and quarters were infused with either *L. lactis* DPC3147 (285LH, 370RH, 400LH, 598LF, 1157LF, 1170LF, 1183LH, 1658RF, 1807LF, 1827RH, 1867LH, 1868LF) or Synulox (285RF, 370RF, 400RH, 598RF, 1157RF, 1157LH, 1170LH, 1183LF, 1807RH, 1807LH, 1867RH, 1868RF). The antibiotic was administered three times, at 12 hour intervals, as per the manufacturer's instructions. The *L. lactis* DPC3147 infusion mixture was administered twice, with a 24h interval between infusions. SCC and standard microbiological analysis were performed before and after infusion, and samples were also taken 7 days post-infusion and 12 days post-infusion.

Results

Treatment of chronic infections

Six udder quarters from 4 cows with a history of chronic infection were selected for treatment. Eighteen hours after infusing the *L. lactis* DPC3147 culture, milk samples were taken from each udder. Samples were taken at intervals up to approximately 30 days post-infusion and bacteria enumerated as described above. Colonies were

identified as *L. lactis* DPC3147 by the production of lacticin 3147. Staphylococci and streptococci were identified on the basis of their characteristic haemolysis on blood agar. In three of the quarters (714RH, 714LF and 96RF), infusion of *L. lactis* DPC3147 was followed by a sharp rise in SCC, and a concomitant reduction/elimination of the pathogen (staphylococci) (Fig. 1A). In three of the quarters (714LH, 700RH and 408RH) the infection persisted despite infusion of the lactococcal culture (Fig. 1B). However, interestingly, in the latter three udder quarters, the lactococcal culture did not appear to colonise the udder quarter (Fig. 1B), whereas in the "cured" quarters, the presence of *L. lactis* DPC 3147 was evident (Fig. 1A). The lactococcal culture did not survive long-term in any of the udder quarters. Somatic cell counts returned rapidly to, and remained at acceptable levels in all quarters.

Treatment of clinical infections

The above results prompted us to investigate the effect of infusing of *L. lactis* DPC3147 into clinically infected quarters. Nine quarters from 9 cows with newly acquired clinical signs of mastitis were treated. After treatment, milk samples were collected daily for up to 14 days and intermittently for up to 55 days. Bacterial cultures were enumerated on ABA or GM17 as described above. In all cases the quality and appearance of the milk improved dramatically following the infusion of the lactococcal culture (Figs 2-9). In some cases, despite the clinical nature of the milk, no pathogen was cultured prior to infusion. Where a pathogen was identified, however, infusion of the *L. lactis* DPC3147 culture resulted in the elimination/ reduction of the pathogen. Pathogens eliminated included *Staphylococcus epidermidis* (Cow 14 LH), *S. aureus* (Cow 1184 RF) non-haemolytic *E. coli* (Cow 1163 RH), and *Strep. uberis* (Cow 1154 LF and Cow 264 LF) (Table 1). In two cases, while treatment resulted in an improved appearance and quality in the milk, the pathogen was not eliminated. These cases included one *Strep. uberis* infection (Cow 1176LH) and one *S. aureus* infection (Cow 1850RF) (Table 1). The data from this trial, including historical data on all the cows used can be viewed in Appendices 1-9.

Immunological studies

The effect of the probiotic *L. lactis* DPC3147 on the immune systems of cows was investigated by analysing leukocyte levels and phenotypes in milk. In an initial

pilot trial, the effect of *L. lactis* on the immune response of two cows was investigated. Both an infected animal and an infection-free cow were used. The results (Fig. 10) indicated that infusion of the *L. lactis* DPC3147, but not the infusion of sterile broth, resulted in a massive recruitment of PMN to the udder, indicating the *L. lactis* may be a specific trigger of the mammary immune response and elicits PMN migration and accumulation. Superoxide anion production assays were also performed and indicated that the newly recruited neutrophils had a higher respiratory burst capacity than resident neutrophils, thus providing the mammary gland with an effective mechanism for the elimination of mastitis pathogens.

In light of these results, a second trial was performed. An uninfected animal (Cow 1803) was chosen to investigate the effects of the *L. lactis* culture. As controls, one quarter (LF) was infused with a lactating-cow antibiotic (Multimast L.C.) and one quarter (RF) was left untreated. A third quarter (LH) was infused with cell-free supernatant from an overnight culture of *L. lactis* DPC3147, and the final quarter (RH) was infused with the diluted *L. lactis* DPC3147 culture. Milk samples were collected pre- and post-infusion and analysed for SCC and differential cell (leukocyte) count. Figure 11 presents neutrophil (PMN) and lymphocyte (CD3) proportions in milk samples before treatment (0 hour) and after treatment (24 and 48 hours). Actual values were calculated using the percentage of positive cells from live/dead flow cytometry analysis and results from the Bentley Somacount Somatic Cell Counter. PMN levels in the control quarter (RF) remained unchanged during the trial period. The probiotic-injected quarter (RH) experienced a dramatic increase in neutrophils over the first 24h period from $2.85x10^2$ cells/ml before treatment to $1.46x10^4$ cells/ml at 24h after treatment (Fig. 11 and Table 2). The supernatant and antibiotic treatments also induced an increase, albeit not as pronounced (from $4.29x10^2$ cells/ml to $1.68x10^3$ cells/ml and $5.5x10^2$ to $1.3x10^3$ respectively), in PMN levels in milk (Table 2). At 48 hours the PMN levels appeared to decrease in the *L. lactis* treated and in the Multimast-treated quarter, but continued to rise in the supernatant-treated quarter (Table 2). Considering these results, it can be concluded that the injection of the *L. lactis* culture resulted in a massive recruitment of PMN to the udder in the 24-hour period following treatment. The culture supernatant also induced a recruitment of PMN to the udder and this was a more sustained induction increasing over 24 hours and continuing to rise, up to 48 hours after treatment. The antibiotic Multimast generated a weaker transient recruitment of PMN to the udder. These results suggest

that both the *L. lactis* culture and the culture supernatant could be specific triggers of the mammary immune response and elicit PMN migration and accumulation. It is possible that the factor responsible for the immune response could be released into the growth medium, which would explain the significant PMN migration in response to the culture supernatant. Levels of lymphocytes were also investigated. The *L. lactis* culture, and the culture supernatant to a lesser extent, triggered an influx of lymphocytes to the udder; the antibiotic, however, did not alter the level of lymphocytes present when compared to the control values (Table 2).

The functional activity of PMNs in the quarter milk samples was investigated for all samples before and after injection. The results of the superoxide anion production assays are presented in Figure 12A and B. The fold increase refers to the proportional increase in superoxide anion production by PMN, from a resting state (T0) to an activated state following activation by phorbol myristate acetate (PMA; incubation for 10 minutes, {T10}). The most obvious activation occurred in the LH quarter which was treated with cell-free supernatant, with a massive activation of neutrophils at 24 hours. Surprisingly, treatment with the *L. lactis* culture did not result in a huge fold activation of neutrophils (Fig. 12A). This can be explained, however, by results of analysis of the fluorescence intensity of all samples (Fig. 12B). The relative fluorescence intensity is a measurement of the fluorescence emitted by the cells; a stronger fluorescence indicates a higher capacity to generate superoxide anion. The resting resident neutrophils in the *L. lactis*-treated quarter (T0) already possessed a very high superoxide anion production capacity (elevated fluorescence intensity) at 24 hours, and, therefore, could not exhibit a marked increase in activation following PMA treatment. In conclusion, *L. lactis* treatment resulted in a massive recruitment of PMN to the udder which were in a highly activated state. The Multimast treatment did not alter superoxide anion production in the treated quarter and the control quarter did not change significantly over the trial period (Figure 12A and B). The PMNs recruited in response to culture supernatant treatment also possessed an elevated superoxide anion production capacity, which appeared to peak over the first 24-hour period following treatment. The results indicated that intramammary treatment with *L. lactis* DPC3147 or with the cell-free supernatant generated from this culture, activated the mammary immune response by triggering the influx of neutrophils to the mammary gland (Fig. 11). These newly recruited neutrophils appeared to possess a higher respiratory burst capacity than resident neutrophils (Fig. 12).

The somatic cell count was monitored every 24 hours up to 7 days following treatment and cell counts are presented in Figure 13 and Table 3. From Figure 13 and Table 3, it is clear that *L. lactis* DPC3147 elicits an enormous cellular response by 24 hours resulting in an elevated SCC, which peaks after 48 hours resulting in a mild clinical infection and gradually drops back to normal over the course of 3-4 days. Treatment with the culture supernatant appeared to elicit a similar response. Our analysis of leukocyte populations and neutrophil activity levels confirm these findings up to 48 hours.

Effects of using dead cells

In order to investigate if viable *L. lactis* DPC3147 were required to produce the immune response generated above, infusion mixtures containing either live or dead cells were prepared and infused randomly into the teats of three cows as outlined in Table 4. Both live and dead cells generated a rise in SCC (data not shown), and, as can be seen from Fig. 14, the dead cells elicited a weak influx of both PMN in each of the cows. An increase in lymphocyte numbers was also observed (data not shown). This recruitment of PMN and lymphocytes in the quarters treated with the dead culture, however, was insignificant compared to the influx in response to the live culture. Thus, it would appear that viable *L. lactis*, but not a killed culture, can specifically elicit recruitment of PMN and lymphocytes to the mammary gland.

Effect of using other LAB

On analysis of results, the question arose as to whether the phenomenon of PMN recruitment was limited to *L. lactis* DPC3147 itself, or if other bacterial strains could also exert this effect. It was decided, therefore, to examine the effect of infusing other food-grade, non-pathogenic bacteria into the udder of lactating cows. A bacterial strain, *Lb. plantarum* DPC4922 was selected on the basis of its evolutionary divergence from *L. lactis* (quite distantly related) as well as the fact that as it was originally isolated from a food source, it can, like *L. lactis* DPC3147, be regarded as a GRAS organism. A third strain, *L. lactis* 5399 (a Bac- derivative of *L. lactis* DPC3147) was also used because of its close similarity to *L. lactis* DPC3147. The infusion mixtures were prepared as described in Materials and Methods and the mixtures were then infused randomly into the teats of three cows as outlined in Table 5. Figures 15 and 16 present neutrophil (PMN) proportions in milk samples from the

three cows over the 10 day trial period. Actual values were calculated using the percentage of positive cells from live/dead flow cytometry analysis and the Somacount readings. The response in the three cows was variable but a similar trend was observed in each case. PMN levels in all the untreated quarters remained relatively unchanged over the trial period (Figure 15). Treatment with *Lb. plantarum* DPC4922 resulted in a slight increase in PMN in all quarters, approaching similar levels to that resulting from *L. lactis* DPC3147 treatment by day 3 in Cow 1181 (Fig. 15). However, the *L. lactis* DPC3147 response in Cow 1181 was somewhat reduced compared to the other two animals (Fig. 15).

Infusion of *L. lactis* DPC3147 in each animal resulted in a dramatic increase in neutrophils in the first 24-hour period after treatment. The Bac- culture (*L. lactis* DPC5399) also induced an increase in all treated quarters, with particularly higher levels of PMN obtained in milk from Cow 1163. However, if the proportional increases in PMN relative to D0 are compared (Figure 16), it can be seen that there is a significant proportional increase in PMN in the *L. lactis* DPC3147-treated quarter compared to the *L. lactis* DPC5399- treated quarter. The PMN influx seems to occur earlier in the *L. lactis* DPC3147-treated quarters (by day 1) compared to the quarters treated with *L. lactis* DPC5399 (Bac-) or *Lb. plantarum* DPC4922 (Fig. 15). In the quarters treated with the latter two treatments, a significant increase was only observed on Day 2 (Fig. 15). Thus, it appears that *L. lactis* DPC 3147 can elicit a stronger and more rapid immune response than either a bacteriocin negative derivative of the same strain or another LAB strain, though the latter strains may also elicit a weaker response.

Comparison of different preparations of L. lactis DPC3147

In order to investigate if different preparations of *L. lactis* DPC3147, other than the standard overnight culture (broth preparation) could produce the immune response generated above, infusion mixtures containing either freeze-dried cells, or the broth culture were prepared. The mixtures were then infused randomly into the teats of three cows as outlined in Table 6. The results (Fig. 17) show an increase in PMN by Day 1 in both treated quarters compared to the untreated quarter in each animal tested. There seems to be a greater influx in two of the over-night culture-treated quarters compared to quarters treated with the freeze-dried culture (Cow 275 and Cow 1134) with a higher number of PMN elicited by the freeze-dried culture in the remaining

animal (Cow 2810). The variation in response is due to the typical variations in immune response between different animals. Thus, both a "fresh" and a freeze-dried preparation of *L. lactis* DPC3147 are capable of eliciting an immune response in the mammary gland.

Comparison of L. lactis DPC3147 treatment with intra-mammary antibiotic treatment

The effects of using *L. lactis* DPC3147 treatment versus using a commonly used antibiotic treatment both for treatment and prevention of intramammary infections caused by *S. aureus* are shown in Table 7. As can be seen in the Table, by Day 7, the *L. lactis* results were very promising, with staphylococci isolated from only two quarters of the 7 quarters originally infected with this organism. In comparison, the quarters treated with Synulox were still shedding *S. aureus* from 6 of the 8 quarters originally infected. However, by day 12, two more of the quarters infused with *L. lactis* were also shedding *S. aureus*, giving a total of 3/7 "cured" by *L. lactis* treatment as opposed to 2/8 "cured" by the synulox treatment. These data indicate that the *L. lactis* DPC3147 treatment is as effective at eliminating infections as the Synulox treatment.

Conclusions

From these results several conclusions may be drawn. Firstly, it is apparent that intramammary infusion of *L. lactis* DPC3147 into cows with chronic infections results in a rapid rise in SCC, often followed by eradication of infection. Additionally, infusion into cows with newly acquired clinical mastitis results in a rapid improvement in milk quality. *L. lactis* DPC3147 treatment has also been shown to be as effective as using a widely used commercial intra-mammary antibiotic in the treatment and prevention of intramammary infections caued by *S. aureus*. It is possible, therefore, that the infusion of *L. lactis* acts as a stimulus which induces release of proinflammatory factors and a prompt recruitment of neutrophils to the mammary gland. The results of immunological studies highlight a number of important findings that may shed some light on the mechanism of action of the

probiotic bacteria *L. lactis* in the mammary gland. These findings include the following:

- Intramammary treatment with either *L. lactis* culture or the culture supernatant activates the mammary immune response by triggering the influx of neutrophils to the mammary gland.
- The *L. lactis* culture and culture supernatant appear to be specific in eliciting recruitment of PMN to the udder, when compared to the antibiotic Multimast L.C.
- These newly recruited neutrophils possess a higher respiratory burst capacity than resident neutrophils thus providing the mammary gland with an effective mechanism for the elimination of mastitis pathogens.
- The factor(s) responsible for eliciting an immune response in the udder may be a soluble factor(s) released into the growth medium, this factor(s) must be either be heat labile or destroyed/utilised rapidly when cells are killed, as dead cells plus supernatant did not elicit an immune response.
- The *L. lactis* culture must be viable to elicit an adequate immune response, although a freeze dried preparation is also effective at stimulating the immune system in the mammary gland.
- Other LAB may also be capable of eliciting an immune response in the mammary gland, in a similar fashion to *L. lactis* DPC 3147, though possibly not to the same extent as L. lactis DPC 3147. This implies that other LAB may also be capable of curing clinical mastitis in dairy cows and other animals if infected quarters were infused with these cultures.
- Treatment with L. *lactis* culture is as effective at eliminating infections as intra-mammary antibiotic treatment.

The words "comprises/comprising" and the words "having/including" when used herein with reference to the present invention are used to specify the presence of stated features, integers, steps or components but does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

Claims

1. Use of a live culture of a non-pathogenic food grade probiotic bacterium in the treatment of infectious diseases.

2. Use as claimed in claim 1 wherein the infectious disease is a localised infection of the skin.

3. Use as claimed in any preceding claim wherein the probiotic bacterium is a non-pathogenic lactic acid bacterium.

4. Use as claimed in claim 3 wherein the lactic acid bacterium is a Lactococcus strain.

5. Use as claimed in claim 4 wherein the Lactococcus strain is selected from the group comprising; Lactococcus lactis DPC3147, Lactococcus lactis 5399, Lb. Plantarum DPC4922.

6. Use as claimed in claim 1 to 5 wherein the disease is a mastitis infection.

7. Use of a freeze-dried preparation of a live culture of a non-pathogenic food grade probiotic bacterium in the treatment of infectious disease.

8. Use of the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium in the treatment of infectious disease.

9. Use as claimed in claim 8 wherein the infectious disease is a localised infection of the skin.

10. Use as claimed in claim 8 or 9 wherein the probiotic bacterium is a non-pathogenic lactic acid bacterium.

11. Use as claimed in claim 10 wherein the lactic acid bacterium is a Lactococcus strain.

12. Use as claimed in claim 11 wherein the Lactococcus strain is selected from the group comprising; Lactococcus lactis DPC3147, Lactococcus lactis 5399, Lb. Plantarum DPC4922.

13. Use as claimed in any of claims 8 to 12 wherein the disease is a mastitis infection.

14. Use of a live culture of a non-pathogenic food grade probiotic bacterium or the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium in the preparation of a medicament for the treatment of infectious diseases of humans and animals.

15. Use as claimed in claim 14 wherein the infectious disease is mastitis.

16. Use of a live culture of a non-pathogenic food grade probiotic bacterium or the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium in a method of stimulation of the immune system.

17. Use as claimed in claim 16 where the live culture or supernatant is used to stimulate PMN cells.

18. A pharmaceutical composition comprising a pharmaceutically effective amount of a non-pathogenic live culture of a food grade probiotic bacterium or a pharmaceutically effective amount of the supernatant of a live culture of a non-pathogenic food grade probiotic bacterium together with a pharmaceutically acceptable carrier or diluent.

19. A method of treatment of infectious diseases comprising administering to a subject a pharmaceutically effective amount of a non-pathogenic live culture of a food grade probiotic bacterium or the supernatant of a non-pathogenic live culture of a food grade probiotic bacterium.

20. A method of treatment as claimed in claim 19 wherein the infectious disease is localised to the skin.

21. A method of treatment as claimed in claim 19 wherein the infectious disease is localised to an infected wound.

22. A method of treatment as claimed in claim 19 wherein the infectious disease is localised to the urinary tract.

23. A method of treatment as claimed in claim 19 wherein the infectious disease is mastitis.

24. Use as claimed in claim 1 whereby there is an accelerated improvement in the quality of milk from cows with mastitis.

25. Use as claimed in claim 1 substantially as described herein with reference to Tables 1 to 7 and Figures 1 to 17 of the Examples.

26. A pharmaceutical composition as claimed in claim 18 substantially as described herein with reference to Tables 1 to 7 and Figures 1 to 17 of the Examples.

27. A method as claimed in claim 19 substantially as described herein with reference to Tables 1 to 7 and Figures 1 to 17 of the Examples.

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